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13. ABSTRACT (Maximum 200 Words)

Breast cancers arise due to an imbalance in cell production relative to cell turnover, resulting in a net accumulation of abnormal cells. Cell turnover is normally achieved through programmed cell death, also known as apoptosis. Defects in apoptosis occur in breast cancers and other types of malignancies, making tumor cells difficult to kill by chemotherapy, hormonal therapy, and radiation. Restoring function of cell death pathways is a strategy for improving treatment of breast cancer.

This project focused on two anti-apoptotic proteins discovered by our laboratory, BI-1 and BI-2 (also known as BAR). The findings provided insights into the mechanism by which these proteins protect cancer cells from specific types of death stimuli. Proof of concept experiments demonstrated that interfering with expression or function of BI-2 (BAR) restores sensitivity of tumor cells to the killing mechanisms employed by immune cells, such as activators of the TNF-family receptor member Fas (CD95). We also observed that BI-1 protects cells from cell death induced by ER stress reagents, and regulates homeostasis of Ca^{2+} in the ER. We found that regulating ER Ca^{2+} homeostasis is a common way for anti-apoptotic proteins such as Bcl-2 and Bcl-xL to protect cells. Green tea compounds that inhibit Bcl-2 can restore normal ER calcium regulation. These findings provide new insights into cell death regulation in breast cancer.

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ANNUAL REPORT

Trainees: Hong Zhang; Han Jun Chae; Can Jin
(Mentor: John C. Reed)

INTRODUCTION

An important reason for breast cancer is the lack of programmed cell death, also known as apoptosis (1). A group of structurally conserved proteins including Bcl-2 and Bax (2), are key players in apoptosis. However, despite decades' effort, how they promote or prevent apoptosis is still not very clear. To better understand this process, our lab screened human cancer cDNA libraries for inhibitors of Bax-induced cell death in yeast. Two proteins were identified: BI-1 and BI-2 (also known as BAR or BARC) (3,4). The purpose of this fellowship was to "investigate the role of BI-2 in regulating apoptosis and the effects of its interactions with Bcl-2 and Caspase-8 on programmed cell death in breast cancer." Another purpose for this fellowship is to compare the anti-apoptotic mechanism of BI-1 and BI-2.

BODY

BI-2 (BAR) is an integral membrane protein mainly localized to endoplasmic reticulum (ER) (4,5). Previously, we showed that the BI-2 (BAR) is predominantly expressed in neurons (brain cells) in vivo (5). We showed that the BI-2 (BAR) interacts with several other DED-containing proteins, including pro-Caspase-8 (4,6), Hip, Hippi, and Bap31 (5). The interactions with the Huntingtin-interacting protein, Hip, and its partner Hippi, have suggested a potential role for BAR in suppressing a cell death pathway of relevance to Huntington's disease (7).

BI-2 (BAR) is also over-expressed in several types of cancers and cancer cell lines, including breast cancer lines such as MCF7 (4, 6), suggesting a link to cancer. In these cancer cell lines, reduced BI-2 (BAR) expression by antisense-RNA sensitizes the cells to Fas involved cell death pathway. Consistently, over-expression of BI-2 (BAR) by transfection rendered cells more resistant to this cell death pathway. In addition, when co-expressed with anti-apoptotic proteins Bcl-2 or Bcl-xL, the BAR protein provides protection from Fas-induced apoptosis under circumstances where neither BAR nor Bcl-2/Bcl-xL alone is adequate (6).

We also studied another Bax inhibitor, BI-1. BI-1 is an ER membrane protein with six predicted transmembrane helices (3). Over-expression of BI-1 protects cells against ER-stress induced apoptosis, as well as Bax-induced apoptosis (8). Homologous BI-1 proteins from other species are also functionally conserved in their ability to prevent cell death induced by certain types of stimuli, including

Bax-induced cell death (9). Using BI-1 knockout mice generated in our lab, we have found that ablation of BI-1 expression sensitize cells to ER-stress agents such as Thapsigargin (ER calcium ATPase inhibitor) and Tunicamycin (ER/Golgi glycosyl transferase inhibitor), induced apoptosis. This pathway involves activation of the Bax protein, where Bax undergoes a conformational change and translocates to mitochondria, inducing downstream steps in this apoptosis pathway. BI-1 over-expression blocks Bax activation induced via this ER-stress pathway, but not when Bax is activated by alternative mechanisms. Consequently, in adult *bi-1*$^{-/-}$ mice, the neurons, fibrablasts, and hepotocytes are more sensitive to ER stress-induced apoptosis. These mice also display increased sensitivity to tunicamycin induced renal tubule, kidney and hippocampal neuron injury, and ischemia-reperfusion caused brain injury (8).

An important pathway for ER to regulate apoptosis is through calcium. If less calcium from ER is dumped into cytosol, the chance to overcome some apoptotic stimuli is better. To analyze if calcium is involved in BI-1 protection against apoptosis, Fura-2 is used as an indicator for cellular calcium concentrations. Thapsigargin blocks calcium import into ER from cytosol, therefore causing calcium leaking into the cytosol and triggering a cell death pathway. In HT1080 cells stably over-expressing BI-1, much less calcium is released into cytosol than observed with HT1080-neo control cells. In contrast, *bi-1*$^{-/-}$ MEF cells release more calcium than the wild-type MEF cells. Taken together, these data suggest that BI-1 can lower resting ER calcium levels (8).

To confirm that the cytosolic calcium change is indeed the result of a difference in ER calcium pools, an ER-targeted, Ca^{2+}-sensitive fluorescent protein called cameleon, was used (10). Indeed, the average resting ER calcium level in HeLa cells transiently transfected with BI-1 is approximately half of that of control HeLa cells (Fig. 1). In addition, co-expression of SERCA2b, the ER calcium pump, restores the basal ER calcium in BI-1 over-expressing cells to the level of the control cells (Fig.1), confirming the authenticity of these results.

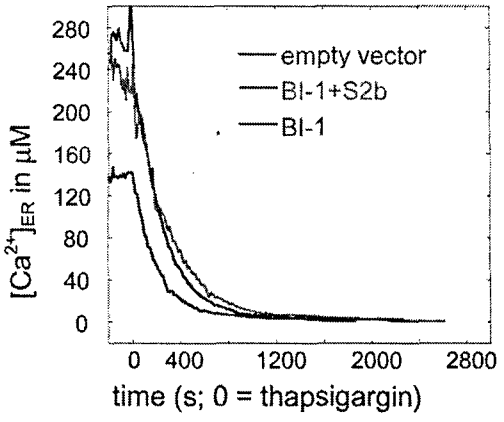

Fig. 1 BI-1 over-expression decreases ER Ca^{2+} levels. HeLa cells were transiently transfected with 1/20 cameleon with pcDNA3-HA or pcDNA3-HA-BI-1, or pcDNA3-HA-BI-1 and pcDNA3-SERCA2B (S2b). Three days later, YFP FRET/CFP fluorescence ratio of cameleon were analyzed in live cells in HBSS media with calcium. The emission ratios were then converted to calcium concentration as described (10). Each trace represents the average of > 20 cells.

To extend these observations regarding Ca^{2+} regulation to Bcl-2-family proteins, we explored the effects of Bcl-2 and Bcl-xL on ER calcium regulation using the same ER-targeted cameleon protein. Bcl-2 is over-expressed in most Estrogen Receptor-positive breast cancers. Bcl-xL is known to be over-expressed in many aggressive Estrogen Receptor-negative breast cancers, conferring resistance to many cell death stimuli. We observed that over-expression of either Bcl-2 or Bcl-xL also reduces basal concentrations of Ca^{2+} in the ER, and thus causes less Ca^{2+} to be released into the cytosol when cells are stressed with agents such as Thapsigargin (figure 2 for example of data). We conclude therefore that BI-1 shares in common with Bcl-2 and Bcl-xL the ability to decrease ER stores of Ca^{2+} thus causing less accumulation of Ca^{2+} in the cytosol when cells are stressed.

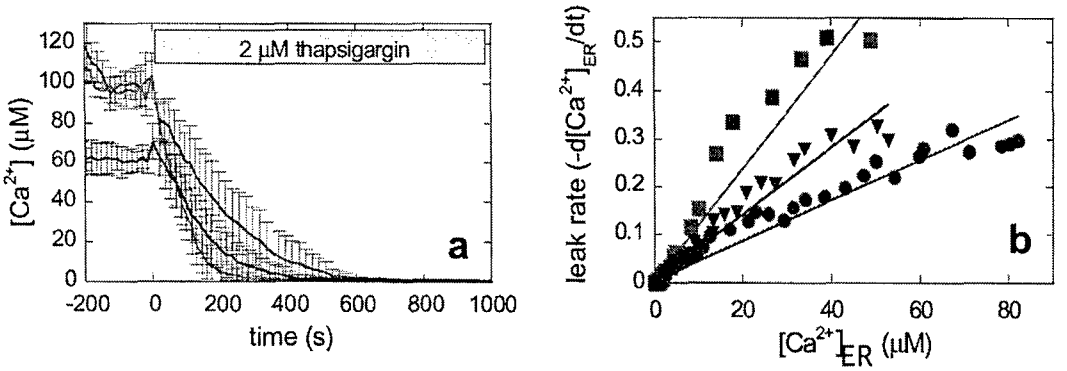

Figure 2. The effect of Bcl-2 overexpression on the $[Ca^{2+}]_{ER}$ of MCF-7 cells. a) Comparison of $[Ca^{2+}]_{ER}$ in neo (red), Bcl-2 (blue), and Bcl-2 cells also overexpressing SERCA 2b (green). Each trace represents the average of > 5 cells. Thapsigargin was added at time t = 0 in the absence of external Ca^{2+} to prevent Ca^{2+} influx due to capacitative Ca^{2+} entry. b) Leakage rate of Ca^{2+} from the ER as a function of $[Ca^{2+}]_{ER}$ for Bcl-2 overexpressing (blue), neo (red), and Bcl-2 cells overexpressing SERCA 2b (green). The leakage rate was determined by taking the derivative of the $[Ca^{2+}]_{ER}$ upon treatment with thapsigargin.

To explore the therapeutic potential of targeting this ER calcium mechanism, we treated MCF7 breast cancer cells with (-) epigallecatechin gallate (EGCG) from green tea that our laboratory previously showed binds to and suppresses the anti-apoptotic mechanisms of Bcl-2 and Bcl-xL. EGCG (but not an inactive compound from the same structural class) restored ER Ca^{2+} concentrations to normal levels in breast cancer cells over-expressing Bcl-2 (Figure 3). We concluded therefore that chemical inhibitors of anti-apoptotic proteins of the Bcl-2 family have the potential to restore normal Ca^{2+} homeostasis, correlating with restoration of tumor sensitivity to apoptosis.

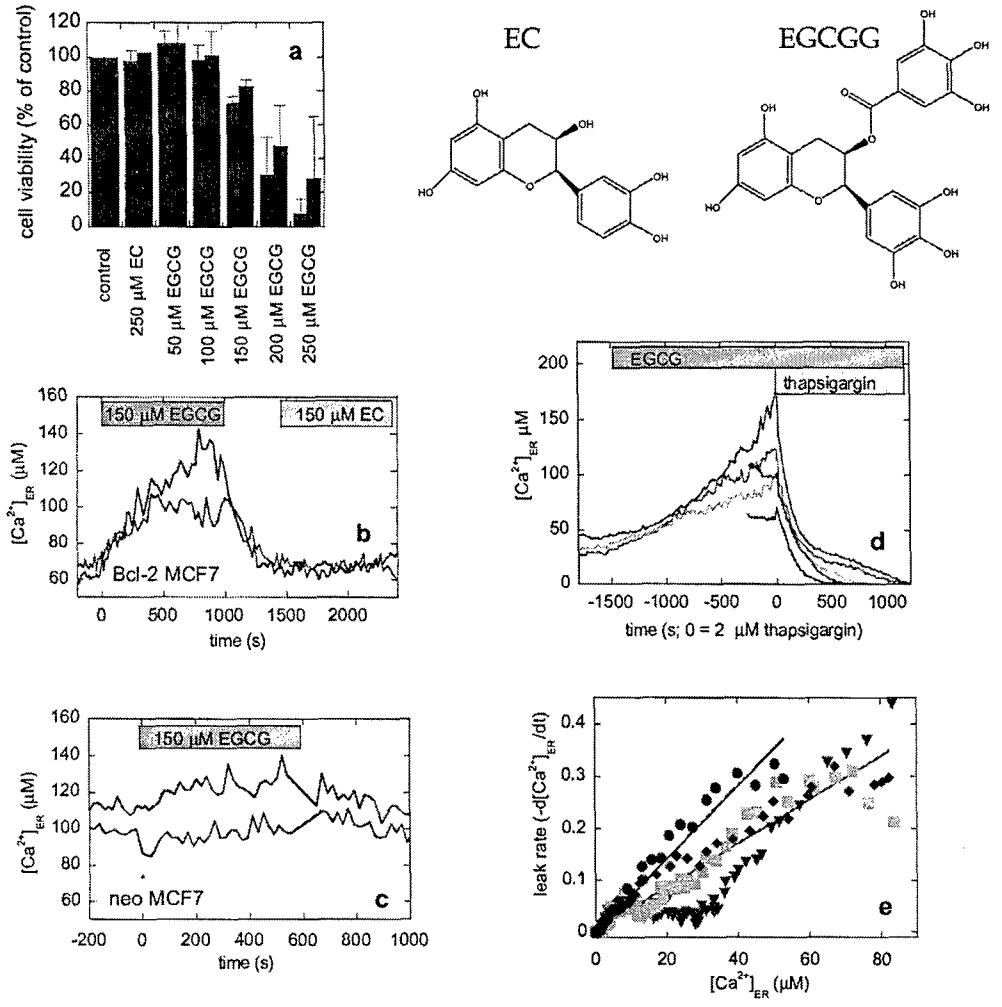

Figure 3. The effect of the green tea compound EGCG and a control compound EC on apoptosis and $[Ca^{2+}]_{ER}$ of MCF-7 cells. The structures of the compounds are shown on the right-hand side of the Figure. a) Cell viability, presented as the % viability compared to the control, as determined by FACS analysis of propidium iodide and annexin V-stained neo (red) and Bcl-2 (blue) cells following treatment with nothing (control), EC, and increasing concentrations of EGCG. Cells were treated for 48 hours with the apoptosis-inducing agents before sorting. b) Treatment of MCF-7 cells overexpressing Bcl-2 with EGCG and EC, as denoted by the green and blue boxes. The traces each represent two different cells. c) Treatment of MCF-7 neo cells with EGCG as denoted by the green box. The traces represent two different cells. It should be noted that EC did not cause an increase in $[Ca^{2+}]_{ER}$ in neo cells. d) Bcl-2 overexpressing MCF-7 cells treated with EGCG (green traces, each representing an individual cell) compared to neo (red) and Bcl-2 (blue) cells. At time t = 0 cells were treated with thapsigargin in the absence of external Ca^{2+} to determine the ER Ca^{2+} leakage rate. e) Comparison of the leakage rates of Ca^{2+} from the ER for Bcl-2 (blue circles), neo (red diamonds), and Bcl-2 cells pre-treated with EGCG (green, upside down triangles and squares), showing the decreased leakage rate upon EGCG treatment.

KEY RESEARCH ACCOMPLISHMENTS

BI-2 (BAR)

- BI-2 (BAR) is an ER membrane protein interacting with other DED-containing proteins and Bcl-2
- BI-2 (BAR) is over-expressed in several cancers and cancer cell lines, including some breast cancers.
- BI-2 (BAR) protects cells against FAS/TNF-induced apoptosis in tumor cells, including breast cancers.

BI-1

- BI-1 is a structurally and functionally highly conserved cytoprotective protein among fungi, plants, and animals
- BI-1 knockout (*bi-1*$^{-/-}$) mice were successfully generated
- Endogenous BI-1 is required to protect cells against ER stress-induced apoptosis
- BI-1-deficient mice display increased sensitivity to ER-dependent tissue injury
- BI-1 over-expression interrupts cell death signaling between ER and mitochondria
- BI-1 regulates resting ER calcium levels
- The effects of BI-1 on ER calcium mimic the effects of anti-apoptotic proteins Bcl-2 and Bcl-xL, which are over-expressed in most breast cancers.
- Chemicals from green tea that bind and inhibit Bcl-2 and Bcl-xL restore normal homeostasis of ER calcium regulation

REPORTABLE OUTCOMES

1. Publications: Trainees working on this grant published 3 papers directly germane to the goals of the grant (4, 8, 9), and published two additional papers indirectly relevant to the goals of this grant (12, 13). Also, trainees associated with this grant wrote two review articles (11, 14). In addition, a manuscript describing the effects of Bcl-2-family proteins and green tea compounds on ER calcium regulation has been submitted (15).

2. Reagents: Multiple reagents were generated as a result of this grant, including many plasmids encoding BI-2 (BAR) and BI-1, and fragments of these proteins containing or missing some of the functionally important domains; antibodies that recognize BAR; antisense oligonucleotides with optimized sequence for suppressing expression of BAR; and cell lines stably expressing transfected BAR and BI-1 or fragments of BAR. BI-1 knockout mice and BI-1 knock-out cells were also generated.

3. Employment. Drs. Hong Zhang and Han Jun Chae who trained on this grant obtained employment. Dr. Zhang worked as a senior research scientist in the division of Oncology Research at Dupont, Inc (Glen Olden, PA) and is now a Project Leader at Conforma Pharmaceuticals, Inc. (San Diego, CA). Dr. Zhang is dedicated to discovery and development of new drugs for cancer, including breast cancer. Her current work focuses on small-molecule inhibitors of Hsp90, a protein over-expressed in many chemorefractory breast cancers. Dr Han Jun Chae is now an Assistant Professor in the College of Medicine, Chonbk National University, in Seoul, Korea. Dr. Chae's work focuses on apoptosis dysregulation in cancer, including breast cancers. She devotes 80% of her effort to cancer research, and also teaches medical students. Dr. Can Jin continues her training in the laboratory.

CONCLUSION

BI-1 and BI-2 (BAR) were identified in our laboratory as inhibitors of Bax lethality. The research supported under the fellowship proved that both of these proteins can protect tumor cells from cell death induced by specific types of stimuli, with BI-1 working predominantly on an ER pathway and BAR working mostly on the TNF/Fas death receptor pathway for apoptosis. These results help to elucidate through which mechanisms tumor cells achieve resistance to stress, allowing them to gain a selective survival advantage relative to normal cells. In addition, we showed that BI-1 phenocopies Bcl-2 and Bcl-xL with respect to regulation of ER Ca^{2+}, suggesting commonalities in their mechanisms. Bcl-2 and Bcl-xL are anti-apoptotic proteins over-expressed in most breast cancers. We demonstrated the chemical compounds from green tea that inhibit Bcl-2 and Bcl-xL restore normal ER calcium regulation, providing insights into the mechanisms of these medicinal products and suggesting paths forward for new therapies.

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